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This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. 1.53 (c).

Docket Number	265.00450160	Type a plus sign (+) inside this box >	+

INVENTOR(s)/APPLICANT(s)

Name (last, first, middle initial)	RESIDENCE (CITY, AND EITHER STATE OR FOREIGN COUNTRY)
Hamill, Owen	Galveston, TX
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TITLE OF THE INVENTION (280 characters max)

METHODS AND COMPOSITIONS FOR BLOCKING METASTASIS PROGRESSION IN TUMOR CELLS

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ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification	Number of Pages	33		Small Entity Statement
	Drawing(s)	Number of Sheets			Other (specify)

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	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT	($) 160
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Respectfully submitted,

SIGNATURE David L Provence

Date January 9, 2004

TYPED OR PRINTED NAME David L. Provence

REGISTRATION NO. 43,022

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PROVISIONAL APPLICATION FILING ONLY

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Applicant(s): Hamill et al.

Docket No.: 265.004501060

Title: METHODS AND COMPOSITIONS FOR BLOCKING METASTASIS PROGRESSION IN TUMOR CELLS

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By: Sara E. Olson
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Patent

File 265.0045 0160

METHODS AND COMPOSITIONS FOR BLOCKING METASTASIS PROGRESSION IN TUMOR CELLS

SUMMARY OF THE INVENTION

We have discovered a stretch-activated, mechanosensitive Ca^{2+} permeable channel (MscCA, also referred to as SACC) in motile tumor cells whose activity is apparently critical for prostate tumor cell migration and thereby may be rate limiting in the metastatic progression of prostate tumors. The identification of a specific molecular target in tumor cells now allows the development of drugs, antibodies and genetic stragtegies to block this channel and thereby block metastatic progression of tumors. In particular, we have shown that agents that target the MscCa including gadolinium ions and a specific peptide present in the venom of the tarantula spider potently blocks migration in the highly motile, metastatic human prostate tumor cell PC3. We envisage the synthesis and refinement of specific peptides that could be used to selectively block the migration and invasiveness of prostate tumor cells in paticular, and solid tumor cells in general.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

a. **Specific Aims**

Our research is based on the emerging concept that dysregulation of cell motility is a critical step in the metastatic cascade leading to migration and invasion by prostate tumor cells. **The long-term goal of our res arch is to identify and characterize mechanosensitive and Ca^{2+}-dependent regulatory mechanisms that are rate-limiting in prostate tumor cell motility.** Cell motility involves a coordinated cycle of events including cell extension, adhesion, contraction and release of rear adhesions. Several Ca^{2+}-dependent mechanisms have been identified that regulate cell motility. Here we focus on the mechanosensory Ca^{2+} permeable channel (MscCa) that transduces membrane stretch into a Ca^{2+} influx, thereby providing feedback between cell extension and downstream Ca^{2+}-dependent mechanisms that release cell adhesions. Based on our preliminary studies identifying the membrane protein that forms a vertebrate MscCa and our demonstration it is more highly expressed in motile versus non-motile prostate tumor cells, **our hypothesis is that MscCa expression regulates prostate tumor cell migration and invasiveness.**

We will pursue three specific aims to test this hypothesis.

In Aim 1, patch-clamp and pressure-clamp techniques will be used to functionally identify and characterize MscCa's properties, including its pharmacology and membrane surface density, in human prostate tumor cell lines with different metastatic potentials — the non-motile, weakly metastatic LNCaP cells vs the motile, highly metastatic PC3 cells.

In Aim 2, the effects of identified blockers of the MscCa on prostate cell migration and invasion will be examined using time-lapse video microscopy and cell migration and invasion assays.

In Aim 3, overexpression and gene silencing by siRNAs of the transient receptor potential channel 1 (TRPC1), a vertebrate MsCaC, will be used to measure the effects of higher or lower MscCa expression on cell migration and invasion. Western blotting will be used to verify changes in TRPC1 expression, and patch-clamp recording will be used to measure changes in MscCa in the transfected prostate tumor cells.

Our project is highly innovative because our identification of a vertebrate MscCa protein provides the first opportunity to determine how changes in its expression may alter the migration and invasiveness of prostate tumor cells. Our team is the best qualified and prepared to complete the specific aims of this project. We have developed recording and stimulating techniques to study MscCa, we have implemented methods for identification and overexpression of hTRPC1, and finally we have established the feasibility of using human prostate tumor cells lines to study how changes in MscCa expression alters tumor cell migration and invasiveness.

Prostate cancer is the most common form of cancer in men, and kills around 40,000 men in the USA each year. In the early stages, the disease stays in the prostate and is not life threatening, but without treatment metastasizes to other parts of the body and eventually causes death. Current therapies are limited to treatment within the prostate; and there is no cure once it has spread. Therefore, a major challenge is to identify the molecular mechanisms that underlie the metastases of prostate cancer. Given that cell migration plays a critical role in this process, MscCa represents an

exciting new prospect as a marker for and a therapeutic target against the spread of prostate cancer.

Abbreviations

Ab, antibody; $[Ca^{2+}]_i$, intracellular $Ca2^+$ concentration; CSK, cytoskeleton; ECM, extracellular matrix; eGFP, enhanced green fluorescence protein; GsMtx-4, *Grammostola spatulata* venom 4 kDa peptide; h, human; MS, mechanosensitive; MscCa, mechanosensory Ca^{2+} permeable cation channels; MTX, maitotoxin; MW, molecular weight; NOMPC, no mechanosensory potential C; OG, n-Octyl-β-glucoside; PMV, plasma membrane vesicle; siRNAs, small interfering RNAs; SOCC, store-operated Ca^{2+}-channels; TRPC-1, transient receptor potential channel 1 protein; *trpc-1*; transient receptor potential channel 1gene; VGSC, voltage-gated sodium channels; X, Xenopus.

b. Background and significance

The mortality of individuals with prostate cancer is a progressive process involving transformation to unlimited cell growth, immortalization to escape the limits of senescence/apoptosis, and the ability to spread and grow at distal sites (invasion and metastasis). Current therapies are limited to treatment within the prostate and there is no cure once the disease has spread. Therefore, treatments are urgently needed to stop the spread of prostate cancer. It is now recognized that tumor cell's ability to migrate plays a critical role in the spread of prostate cancer (Banyard & Zetter, 1999) – a tumor cell must migrate from the prostate, pass through blood vessels, penetrate the secondary tumor site (typically bone), and migrate through the tissue to establish a metastatic site. Metastasis will only occur if the tumor cell completes every step in this cascade. Thus, identifying the most sensitive dysregulated step in the transformation of non-motile into motile tumor cells should provide the most promising therapeutic target against prostate tumor cell spread and metastasis. As a consequence, there is great interest in understanding the rate-limiting processes in cell motility (Sheetz et al., 1999; Kassis et al., 2001; Wells et al, 2002). In particular, the regulatory molecules that are specific to the process of cell motility must be identified.

Cell motility is also critical for a variety of physiological processes including embryogenesis, inflammation and wound healing. Although motile cells vary in the exact details and rates of their movement (ranging from < 1 μm/min for fibroblasts and tumor cells to ~30 μm/min for fish epithelial keratocytes), they appear to share common features (Lauffenburger & Horwitz, 1996). For example, the traction forces exerted by motile cells are transmitted to the underlying substrate through nascent focal adhesions most likely formed by integrins linked to the actin cytoskeleton. Furthermore, cell movement depends upon the ability of the focal adhesions to assemble and disassemble in response to changes in locally applied force. Therefore, a key question in cell motility concerns the nature of the mechanosensitive (MS) processes that regulate the assembly and disassembly of focal adhesions. In several migrating cells, spatial and temporal gradients in $[Ca^{2+}]_i$ have been demonstrated, with lower $[Ca^{2+}]_i$ in the front that would favor adhesion assembly, and higher $[Ca^{2+}]_i$ in the rear that would promote adhesion disassembly and release (Schwab, 2001). Based on inhibition studies, several Ca^{2+}-dependent molecules have been implicated as participating in adhesion disassembly, including calcineurin, a Ca^{2+}/calmodulin-activated phosphatase, and calpain, a Ca^{2+}-activated cysteine protease (Geiger & Bershadsky, 2001; Webb et al., 2002). However, an exciting new candidate is the mechanosensory Ca^{2+}-permeable channel(s) (MscCa) that transduces membrane stretch into Ca^{2+} influx, thereby providing positive feedback between cell extension and the Ca^{2+}-dependent disassembly mechanisms. For example, in the highly motile fish epithelial keratocyte, Lee et al (1999) have used Ca^{2+} imaging and patch-clamp recording, to demonstrate that transient increases in $[Ca^{2+}]_i$ coincide with phases of increased MscCa activation associated with cell extension. Furthermore, Gd^{3+}, a commonly used MscCa blocker, abolishes Ca^{2+} transients and prevents rear detachment, thereby inhibiting cell migration (Lee et al., 1999). The MscCa belongs to the class of MS channels (Sachs & Morris, 1998; Hamill & Martinac, 2001) and is the focus of this project.

Since many studies indicate the mechanism of cell motility is conserved, an attractive hypothesis is that MscCa activity may also be rate-limiting in prostate tumor cell migration. In this case, dysregulation of MscCa expression could transform non-motile prostate tumor cells into motile cells, thereby contributing to tumor cell spread and potential metastasis. However, changes in the expression, or coupling with, a downstream Ca^{2+} signaling mechanism(s) could also be rate-limiting. Therefore, to support a critical role for the MscCa, several issues need to be addressed. **First**, it needs to be shown with patch-clamp study that changes in functional MscCa expression (membrane density and/or single channel properties) correlate with the increased migratory capacity of prostate tumor cells. The availability of prostate tumor cell lines that demonstrate enhanced migration and invasiveness in a stable pattern (e.g., PC-3 vs LNCaP cell lines) provides an opportunity to make this correlation (Zheng et al., 1999; Slack et al., 2001). **Second**, the membrane protein(s) forming the MscCa(s) needs to be identified. Only with this knowledge can changes in MscCa expression, subcellular localization, and interactions with other membrane proteins be measured under genetic or epigenetic conditions that might lead to acquisition of increased tumor cell migration.

On the first issue, we could find no patch-clamp reports describing MscCa in prostate tumor cells (see Section C). This oversight is a critical gap, since other channels, including voltage-gated Na^+ channels (VGSC) and store-operated Ca^{2+} channels (SOCC), have been studied in prostate tumor cells (Diss et al., 1998; Gutierrez et al., 1999). Furthermore, enhanced VGSC expression has been correlated with increased tumor cell invasiveness, while tetrodotoxin, a VGSC blocker, has been shown to reduce invasiveness (Grimes et al., 1995; Smith et al., 1998). Invasiveness is measured *in vitro* by the ability of tumor cells to actively migrate through a basement membrane matrix (e.g., Matrigel), and also depends upon adhesion receptors for matrix attachment and metalloproteinases for matrix lysis. It is unclear at this stage how the VGSC is activated in prostate tumor cells. One interesting possibility is that it occurs via MscCa-induced membrane depolarization. However, a pharmacological study attempting to address the role of MscCa, reported that Gd^{3+} enhanced rather than inhibited prostate tumor cell motility (Verall et al., 1999). Although this result appears to question the generality of the earlier epithelial keratocyte result (Lee et al., 1999), the effects of Gd^{3+} on prostate cell motility were unfortunately measured in a medium containing serum, which includes components that avidly bind Gd^{3+} and reduces its blocking efficacy (Hamill & McBride, 1996; Caldwell et al., 1998). Furthermore, apart from being a nonspecific channel blocker, Gd^{3+} has also been reported in some cells to have concentration-dependent biphasic (stimulatory and inhibitory) effects on MS channels (Hamill & McBride, 1996). These concerns indicate the necessity of directly measuring Gd^{3+} effects on both MscCa and prostate tumor cell migration under the same conditions as will be done in Specific Aims 1 and 2. In addition, because a more potent and specific MscCa blocker (GsMtx-4) has recently been purified from the venom of the tarantula *Grammostola spatulata* (Suchyna et al., 2000), its effects will also be tested.

On the second issue of protein identity, the MscCa can be considered a member of the broad family of Ca^{2+}-permeable channels that may display either strong or relatively weak Ca^{2+} selectivity, and includes voltage-, receptor- and mechano-gated channels as well as the SOCC (Clapham, 1995). While the membrane proteins

forming voltage- and receptor-gated channels have been identified, the protein identity and relationship between MscCa and SOCC remains unclear. For example, members of the transient receptor potential channel (TRPC) family, which includes *Drosophila* channels (TRP and TRP-like) and several vertebrate homologs (TRPC 1-7), have been proposed to form SOCC (Montell, 1997). However, although some TRPCs are sensitive to Ca^{2+}-store depletion and may contribute to store refilling, TRPCs typically display a relatively large single channel conductance (30-80 pS) and are only weakly Ca^{2+} selective, unlike the low conductance (~ 1pS) and high Ca^{2+} selectivity, first described for SOCC in blood cells (Hoth & Penner, 1992; Harteneck et al., 2000), but similar to MscCa (Hamill & McBride, 1996). Furthermore, a distantly related TRPC family member (NOMPC) has been shown to be a mechanically gated channel (Walker et al., 2000). Finally, overexpression of hTRPC1 in *Xenopus* oocytes and in rat liver cells does not increase the endogenous SOCC current, but does increase a Ca^{2+}/cation-conductance that is activated by the marine poison maitotoxin (MTX) (Bereton et al., 2000; 2001). Signiifcantly, the MTX-activated conductance shares basic properties with the oocyte MscCa, including cation selectivity, divalent cation block and pharmacology (Bielfeld-Ackermann, 1998; Weber et al., 2000; Diakov et al., 2001). As described in Section C, we have used detergent solubilization and functional reconstitution of oocyte membrane proteins to show that an ~80 kDa membrane protein identified as TRPC1 is concentrated in the most active protein fraction that reconstitutes MscCa activity. This independent line of evidence adds strong support to the idea that TRPC1 is MscCA and provides the first opportunity to alter MscCa levels by heterologous expression of TRPC1 in prosate tumor cells in order to study their effects on cell migration as will be done in Specific Aim 3.

Evidence indicates that TRPC1 can co-assemble to form a homotetrameric channel (Hofmann et al., 2001). However, there is also evidence for heterotetrameric subunit assembly that may provide the basis for specific MscCas with distinct properties. For example, there are splice variants of TRPC1 (i.e., TRPC1α and TRPCβ) (Engelke et al., 2002; Ong et al., 2002; see preliminary results) and other TRPCs (e.g., TRPC4 and/or TRPC5) that can co-assemble with TRPC1 (Hofmann et al., 2001). Furthermore, several TRPC-related Ca^{2+} channel family members have been identified that are upregulated in prostatic cancers according to their malignancy (Tsavaler et al., 2001; Peng et al., 2000; Wissenbach et al., 2001). Together these observations raise the exciting possibility of "cancer-specific" MscCa that may subject to specific inhibition (e.g., by drugs, Abs or gene therapy). If this also allowed the selective inhibition of tumor cell motility, then the disruption of physiologically important functions (e.g., immunological surveillance and wound healing), associated with general inhibition of cell motility, could be avoided. However, Ca^{2+} channels have been implicated in regulating other steps in cancer progression, including cell proliferation and apoptosis. Clearly, the properties of Ca^{2+} channels in prostate cells and their functions need to be characterized. In particular, single channel patch-clamp studies of MscCa in prostate tumor cells are urgently required, and are part of Specific Aim 1.

Our project represents the critical first step in the functional and molecular characterization of MscCa in prostate tumor cells, and of its role in tumor cell migration and invasion. We have assembled a team that is highly qualified and well prepared to carry out the aims of this project. The PI has been directly involved

in the development of techniques for stimulating and recording of MscCa and is fully familiar with the methods of analysis and the potential artifacts associated with recording MscCa in cells and after reconstitution in liposomes (Hamill et al., 1981; McBride & Hamill, 1992; 1995, Hamill & McBride, 1992, 1995; 1997; Zhang & Hamill 2000a,b; Zhang et al., 2000; Hamill & Martinac, 2001; Martinac & Hamill, 2002). Dr. Rosario Maroto, a trained molecular biologist and Co-PI on the project, has provided the first independent evidence that TRPC1 forms a vertebrate MscCa and demonstrated that TRPC1 is more abundantly expressed in PC3 vs LNCaP cells. Finally, Dr. Miriam Falzon, an expert in cancer biology, and collaborator on this project, has already assisted our laboratory in establishing the human cell lines to correlate MscCa expression with tumor cell metastatic potential and will collaborate further in our transfection experiments (Shen & Falzon, 2003a,b; Tovar Sepulveda & Falzon, 2003).

c. **Preliminary Results**

Protein id ntity of MscCa

The vertebrate MscCa is one of the last of the major classes of functional channels that remains to be structurally identified. Expression cloning strategies have been unsuccessful, presumably in part because the *Xenopus* oocyte, the most commonly used expression vehicle, expresses its own MscCa. We thus followed a different identification strategy, based on detergent solubilization of oocyte membrane proteins followed by functional reconstitution in liposomes, and using patch-clamp recording as a MscCa assay (Sukharev et al., 1994). The feasibility of this approach came with the demonstration that oocyte MscCa activity is retained in CSK-deficient plasma membrane vesicles (PMVs) and after fusion of PMVs with azolectin liposomes (Zhang et al., 2000, Fig. 1, underlined references are from our lab.). The channel conductance (~ 40 pS), multiple reopenings within an event, and stretch sensitivity (i.e, activated at ~20 mmHg) are similar to those of the MsCaC recorded under similar conditions from the oocyte (Zhang et al., 2000).

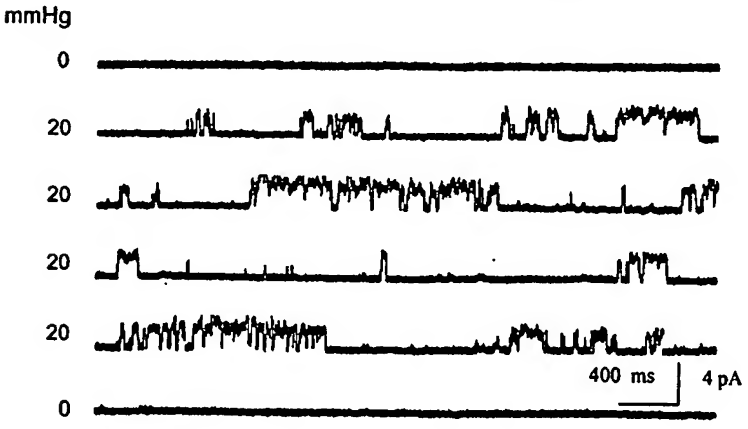

Figure 1. MscCa activity recorded from azolectin liposomes fused with oocyte plasma membrane vesicles. Patch current traces before, during and after the application of 20-mmHg suction to the patch pipette interior. 200 mM CsCl. Patch potential 60 mV.

In the next step (in collaboration with Dr. Boris Martinac, University of West Australia) we solubilized oocyte membrane proteins using n-octyl-β-glucoside (OG) and partially purified proteins using FPLC (Fig. 2). Our patch-clamp assay of the reconstituted fractions indicated no channel activity in fractions 1,2,15-17 (protein-lipid ratio of 1:50), whereas activity was consistently detected in fractions 8-10 (20 of 20 patches). Of the active fractions, only fraction 9 expressed channel activity when the protein-lipid ratio decreased to 1: 5,000 (9 out of 20 patches).

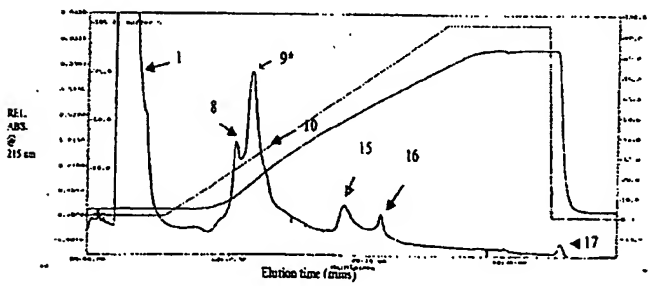

Figure 2. FPLC profile of OG-solubilized membrane proteins. The numbers indicate with arrows the peaks where fractions were collected. The linear gradient is the % buffer (0 –1 M NaCl). Buffer conductivity as a function of elution time is also shown. (Full size version provided in Appendix)

Figure 3A shows a silver-stained gel of the proteins from the FPLC fractions indicated in Fig. 2. The major difference between the active and inactive channel fractions, was the presence of a band running with a molecular mass of ~80 kDa. Based on previous cloning and immunological studies, an ~80 kDa oocyte membrane protein has been identified as TRPC1 (Bobanovic et al., 1999; Brereton et al., 2000, see Section b). We therefore tested and confirmed using Western blots that the ~80 kDa protein concentrated in fraction 9 was TRPC1 (Fig. 3B).

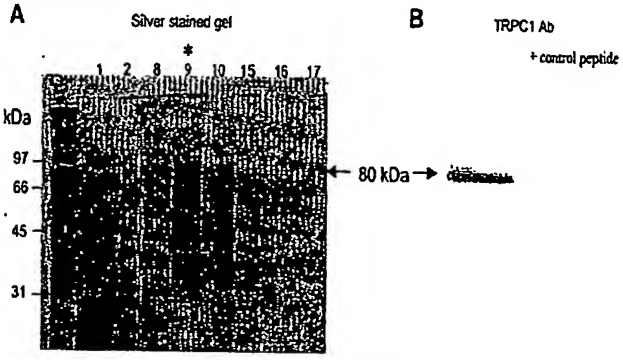

Figure 3. *The ~80 kDa protein that predominates in active FPLC fraction 9 is TRPC1. A:. a silver - stained gel of proteins from the different FPLC fractions in Fig. 2. B: Western blot using a TRPC1 Ab against the proteins in fraction 9, in the absence (left lane) and presence (right lane) of the control peptide.*

We have inserted the cDNA encoding hTRPC1, provided by Dr. Craig Montell (Johns Hopkins School of Medicine), into a dual-function vector, pXOOM, that allows expression in both *Xenopus* oocytes and mammalian cells (Jespersen et al., 2001). In ongoing experiments we are overexpressing hTRPC1 in *Xenopus* oocytes, followed by membrane protein purification and functional reconstitution, to verify that hTRPC1 alone is sufficient to form the MscCa when reconstituted in liposomes.

Prostate tumor cells

We have carried out preliminary studies to establish the feasibility of using PC3 and LNCaP cell lines to study the role of MscCa in cell migration. Figure 4 illustrates the distinct morphology of LNCaP and PC3 cells.

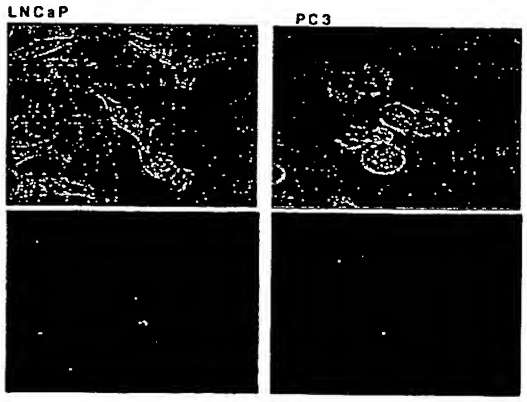

Figure 4. *PC3 and LNCaP tumor cells display distinct morphologies. The top panels are transmission and the bottom panels confocal images. Cells were stained with FM-464 to labe l the membrane and dextran-fluorescein (MW 10,000) to label the endocytotic compartment. (Full size version provided in Appendix)*

Our time-lapse video microscopy studies (i.e., frames at 5 min intervals over 3 h;

cells maintained at 37°C, M3) confirm that of PC3 cells are highly motile compared with LNCaP cells. Our migration assays (carried out in collaboration with Dr. Falzon's laboratory, M3) of PC3 and LNCaP cell migration, as a function of serum concentration, confirm that PC3 cells show greater unstimulated migration (i.e., in the absence of serum) than LNCaP cells, but this difference is reversed in the high serum (see Slack et al., 2000).

% of migration	0% FBS	1% FBS	3% FBS	10% FBS
PC3	29.35%	32.38%	29.9%	13%
LNCaP	17.41%	20.19%	23.29%	27%

Table 1. *Prostate tumor cell migration as a function of serum concentration(see Sect. d, M3)*

The serum-free result is important, because it indicates the feasibility of measuring Gd^{3+} effects on cell migration in the absence of serum. However, in these initial experiments, cell migration was measured through 8 μm diameter pores, which may be a limitation. Therefore, we will also measure migration through 12 μm pores will.

Figure 5A shows a Western blot of OG-solubilized membrane proteins isolated from PC3 and LNCaP cells and from *Xenopus* oocytes. The same TRPC1 Ab that identifies a ~80 kDa oocyte protein identifies a ~100 kDa prostate membrane protein, which is more abundant in PC3 cells compared with LNCaP cells. Figure 4B shows our patch-clamp recordings from LNCaP and PC3 cells in response to ramps of suction applied to the cell-attached patches. Our results are significant because they show for the first time that MS channels are present in human prostate tumor cells, and may be more highly expressed in PC3 than in LNCaP cells. Other interesting features are the apparent channel closing while the ramp is increasing (i.e, adaptation) and the sustained channel activity after the ramp is turned off. Step-changes and steady-state changes in suction/pressure will be used to analyze channel dynamics and open channel properties in the different cell lines (Sect. d).

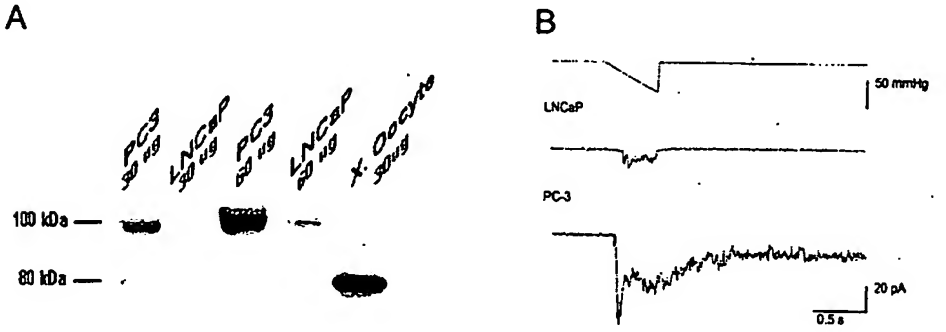

Figure 5. *TRPC1 expression and MS channel activity in PC3 and LNCaP cells. A: Western blot identifying TRPC1 as a membrane protein of ~ 100 kDa more abundant in PC3 vs LNCaP cells and a ~80 kDa protein in oocyte membrane. B. MS currents recorded in LNCaP and PC3 cells using paired patch pipettes. (patch potential –70 mV; pipette solution in mM: 140 KCl, 10 Hepes, 0 Ca^{2+}).*

d. Research Design and Methods

Research Design

Briefly, our three specific aims involve 1) the patch-clamp characterization of MscCa properties in prostate tumor cell lines with different metastatic potentials; 2) determining the effects of MscCa blockers on prostate tumor cell migration and invasion; and 3) determining the effect of modulating MscCa's level, by overexpression and gene silencing of *Trpc-1*, on prostate tumor cell migration and invasion. The results of these experiments will test our hypothesis that **MscCa expression is rate-limiting in prostate tumor cell motility.** Experimental support for this hypothesis should provide a new therapeutic target that is urgently needed to prevent metastasis of prostate cancer.

AIM 1: Patch-clamp/pressure-clamp characterization of functional MscCa expression in human prostate tumor cell lines with different metastatic potentials

Our preliminary studies indicate LNCaP and PC3 cells express MS channels. In this case, differences in cell motility and migration could arise through differences in channel density and/or in single channel properties. Below we describe experiments designed to distinguish between these possibilities.

1a Open channel properties

The first question to be addressed is whether prostate tumor cells express a single homogeneous class or several classes of MS channels. To make this distinction we will first measure open channel properties (e.g., see Bormann et al, 1997). Single channel current amplitude histograms as a function of pressure and voltage will be constructed. The conductance and ion selectivity of the channel states will be determined by measuring reversal potential and single channel current-voltage relations as a function of ion concentration changes and ion substitutions. The results of these experiments should reveal if MS channels in prostate tumor cells display properties similar to the MscCa measured in the *Xenopus* oocyte and mammalian cells: high cation-selectivity ($P_{Cl}/P_K < 0.005$); weak selectivity for the inorganic cations ions ($K^+ > Na^+ > Ca^{2+} \sim Mg^{2+}$); permeant channel block by Ca^{2+} and Mg^{2+}; and intrinsic inward rectification (Guhray & Sachs, 1984; Yang & Sachs, 1990; Hamill & McBride, 1992; 1996). Any differences will be significant because they may reflect differences in the protein composition (e.g., TRPC subunit makeup) of the tumor cell channel(s), and thus provide a basis for selective pharmacological and genetic blocking strategies.

1b Channel density

The membrane patch density of the MS channel(s) will be determined using fast steps of increasing suction/pressure to maximally activate the channels in the patch before adaptation to sustained stimulation (Hamill & McBride, 1992) or by ramps of increasing pressure/suction to measure saturating responses (see Fig. 5B in section c). Paired pipettes (i.e., from a single capillary pull) will be used to compare patch density of the MS channels on PC3 and LNCaP cells and on different regions of the same cell. Several patches will be sampled from different areas on the same cell. Whole-cell MS conductance changes will be measured, by reversibly inflating or by

extending/pulling on the cell while recording current in the whole cell configuration (Morris & Horn, 1990; Hamill & McBride, 1993). Whole cell membrane capacitance measurements will be used to measure the membrane surface area directly from the capacitance compensation on the EPC-7 patch clamp amplifier and assuming 1 $\mu F/cm^2$ (Hamill et al., 1981; Hille, 1984). Whole-cell MS current activation may depend upon the ability of cell inflation or cell stretching to use up excess membrane area reserves (e.g., in the form of membrane folds and microvilli) to increase membrane tension and activate the channel, without inadvertently and irreversibly damaging the cell (Morris & Horn, 1990, Zhang & Hamill, 2000a,b).

1c Gating properties

Both stationary and dynamic gating kinetics, and tension- (pressure-) sensitivity of the channel gating in prostatic cells will be characterized in gently sealed cell-attached patches. Specific interest will be in whether PC3 and LNCaP cells show differences in membrane-CSK interactions as reflected by the channel dynamics and sensitivity to mechanically-induced changes in channel dynamics. In other cells MscCa activity is often characterized by rapid adaptation to sustained pressure pulses, and this dynamic behavior is lost when membrane-CSK interactions are decoupled (Hamill & McBride, 1992; 1997; Small & Morris, 1994). If adaptation can be abolished by mechanical overstimulation, the stationary channel kinetics in the two cells lines will be measured. For example, the MscCa in several cell types shows characteristic burst kinetics (i.e., multiple openings within a single event, see Fig. 1, section d). This behavior is inconsistent with a simple two-state channel (i.e, with 1 open and 1 closed state). However, it can be modeled by assuming 2 open and 3 closed times in which applied pressure (p) increases the open probability (P_o) by reducing the average lifetime of one closed state (Yang & Sachs, 1990). The tension (pressure) sensitivity of the P_o of the MS channels can be described by a Boltzmann $P_o/(1 - P_o) = \exp [\alpha(p - p_{1/2})]$ where $p_{1/2}$ is the pressure at which the channel is open half the time, and α is the slope of the plot $\ln [P_o/(1 - P_o)]$. The Boltzmann parameters are useful in comparing the tension sensitivity of the MscCa in membranes but they also include the effects of different degrees of membrane-CSK coupling (Sachs & Morris, 1998; Hamill & Martinac, 2001). For these measurements paired pipettes will also be used to ensure the diameter and shape of patch pipette tips remain nearly constant throughout the comparisons.

1d Voltage sensitivity

The voltage-sensitivity of MS channels is a potentially important parameter in relationship to cell motility because motile cells, including fish keratocytes and prostate cancer cells, show galvanotaxis (i.e., voltage-directed movement) that has been related to increased influx of Ca^{2+}, possibly via interaction between VGSC and the MscCa (Brust-Mascher & Webb, 1998; Djamgoz et al., 2001). In oocytes, membrane depolarization increases the average open time (i.e, burst duration) of the MscCa activated at constant pressure (Yang & Sachs, 1990, Lane et al., 1991). This voltage effect appears intrinsic to the protein because it is retained in CSK-deficient PMVs (Zhang et al., 2000). It has also been reported that membrane hyperpolarization or depolarization can activate the MscCa at zero pressure (Silberberg & Magleby, 1997; Hamill & Martinac, 2001). The latter effect appears to be dependent on factors extrinsic to the channel protein (e.g., electro-curvature or -

compression effects) because they can be removed by membrane blebbing (Zhang et al., 2000).

1e Pharmacology

An increasing number of ions, drugs and toxins have been discovered that block or activate MS channels (Yang & Sachs, 1989; Lane et al, 1991; Suchyna et al., 2000; see Hamill & McBride, 1996 for review). Determining the specific pharmacology of MS channels in prostate tumor cells is the first step in designing new strategies to block the channel function(s). Here we focus on three reported MscCa blockers. First, Gd^{3+} will be tested, because a previous study has reported Gd^{3+} stimulates prostate tumor cell migration (Verall et al., 1999, but see Sect. b for discussion). Gd^{3+} concentration-response relationships (in the absence of serum) will be measured with particular emphasis on determining whether Gd^{3+} shows a biphasic (excitatory/inhibitory effects) or a monotonic blocking effect. Second, amiloride and its analogs (dimethylamiloride, bromhexamethyleneamiloride and benzamil) will be tested because they show an increasing potency in blocking the oocyte MscCa that can be used to "fingerprint" MscCa -dependent functions (Lane et al., 1992). Third, GsMtx-4, a relatively small (Molecular mass ~ 4 kDa) peptide found in the venom of the tarantula *Grammostola spatulata*, will be tested because it is the most specific and potent blocker of the MscCa yet found (Suchyna et al., 2000). Both patch and whole-cell recording of MS currents will be used to characterize blocker sensitivity.

Expected outcomes and interpretations

Our preliminary studies indicate the presence of MS channels in human prostate tumor cells. The detailed channel analysis proposed here will quantify differences in channel density, open channel properties, gating mechanisms and pharmacology. Our results will also indicate whether the channels are similar to those reported for the MscCa in other cells, most notably the *Xenopus* oocyte. Identical properties would be consistent with the same protein structure (e.g., TRPC1 homotetramer). Significant differences would provide the first clue of differences in protein composition (e.g., heterotetramers with TRPC1 splice variants, different TRPCs or TRPC-related family members) and provide the basis for designing drugs and other strategies to more selectively alter channel activity and their functions in prostate tumor cells.

Pitfalls and alternative approaches

Based on our preliminary studies, we foresee no major pitfalls that would prevent completing this aim. However, the possibility exists that a significant variation in MS channel properties within cells of the same cell line could prevent recognition of differences between cell lines. To reduce/alleviate this source of variation, we will select cells of homogeneous morphology and if necessary generate subclones of the cell lines (e.g., by growing cells in medium of reduced serum). We will also compare MS channels in individual cells that demonstrate differences in motility (i.e., as reflected by prior time-lapse measurements).

Significance

Although several voltage-gated and Ca^{2+}-sensitive channels have been described in human and rat prostate tumor cells, there has been no description of MS channels. This oversight represents a significant gap in knowledge, given that the MscCa is

proposed to be rate-limiting in cell migration, possibly including tumor cell migration. The channel density, open channel and gating properties are critical parameters that must be determined in order to model how the Ca^{2+} channel may modulate the spatio-temporal fluctuations in $[Ca^{2+}]_i$. Finally, as described in the next Aim, knowledge of the pharmacology, in particular dose-response relationships of MscCa blockers, must be available before screening their effects on cell motility and cell migration.

AIM 2: The effects of MscCa blockers on prostate tumor cell migration and invasion.

2a Effects on cell viability

Since migration and invasion assays are carried out over 24 h periods or longer, any apparent reduction in cell migration and invasion caused by MscCa blockers could arise because of differential effects on PC3 and LNCaP cell viability. Therefore, the effects of the agents will first be studied on cell viability including effects on necrosis, which will be monitored by trypan blue and/or PI nuclear staining and effects on apoptosis, which will be monitored by Annexin V-FTIC/negativePI staining (Sect. d, M7). Because migration and invasion assays will be carried out in different serum concertaions, resulting effects on prostate cell viability will also be determined.

2b Effects on cell migration and cell invasion

Dose-response relationships will be determined for the active drugs on prostate tumor cell migration and invasion using the quantitiative fluorescence assays in uncoated and Matrigel coated migration chambers, respectively (Sect. d, M3). Our rationale for testing effects on invasiveness as well as migration is that any induced changes in cell motility may be exaggerated in the invasion assay because the cells have to migrate through an additional barrier. The effects on migratory capacity of PC3 and LNCaP cells, will be determined as a function of serum concentration (see Table 1 in Sect c; Slack et al., 2001). Gd^{3+} effects will only be studied in the absence of serum. In separate experiments, the effects on cell motility of individual cells will be studied with time-lapse videomicroscopy in a 37°C heated tissue culture chambers fitted on the microscope stage.

Expected outcome and interpretation

According to our hypothesis we expect that agents that block the MscCa should also block or reduce cell migration and invasion. In the case, that blockers do not inhibit or stimulate migration (Verall et al. 2000), we would look for side effects, as reflected by changes in cell morphology or adhesion, in addition to changes in cell viability. However, given the diverse agents that inhibit MscCa it would be surprising if they shared common side effects. Effects on migration and invasion will be compared with effects with individual cell motility measurements.

Pitfalls and alternative strategies

If it turns out specific agents do reduce cell viability during the time course of migration and invasion assays, our more acute measurements of cell motility using time-lapse video microscopy (i.e., over 1-3 h) should be less sensitive to toxic effects due to longer term exposures.

Significance

The development of new field of medicine known as channelopathies has resulted in increasing interest and success in targeting specific channels, including several classes of Ca^{2+} channels, that are abnormally expressed/regulated in human disease states (Ashcroft, 1999). Until recently MscCa had a rather limited pharmacology and the commonly used blockers, Gd^{3+} and amiloride, were nonspecific and/or unsuitable for clinical application (Hamill & McBride, 1996). However, the newly recognized MscCa blocker peptide GST-X4 is more potent and specific and may provide the basis for designing new drugs that selectively target MscCa (Suchyna et al., 2000). The results of this specific aim should demonstrate whether these agents might be useful in blocking prostate tumor cell spread and thereby increase the therapeutic window in which other treatments can be used to reduce tumor cell number (i.e., by stimulating apoptosis and/or blocking proliferation). In this way, the MscCa channel would represent an exciting new target in combination therapy to reduce the metastatic potential of prostate tumors.

AIM 3: Overexpression and gene silencing of TRPC1 expression in prostate tumor cells of different metastatic potential.

To directly test the hypothesis that regulation of MsCaC expression can be rate-limiting in tumor cell migration, we will use our recent demonstration that TRPC1 forms a vertebrate MsCaC to genetically up-regulate and down-regulate the expression of a MsCaC in PC3 and LNCaP cells. First, we will test whether overexpression or gene silencing (by siRNAS) of TRPC1 (Sect. d, M5 & M6) alters the viability of prostate tumor cells by increasing apoptosis/necrosis compared with the control cells (i.e., those exposed to LipofectAMINE alone). Second, we will verify with Western blotting the expected changes in TRPC1 membrane protein expression in the transfected cells. Third, we will use patch-clamp recording to examine changes in single channel expression in the transfected cells. The basic questions addressed in this aim will be whether the heterologously expressed MscCa is similar or different in its properties from the endogenous MscCa(s), and whether gene silencing of TRPC1 result in the disappearance of the endogenous MscCa. Answers to these questions will be useful in directly implicating TRPC1 as a structural component of the MscCa in prostate cells. Finally, we will examine the effects of overexpression and gene silencing of TRPC1 on cell migration, invasion and individual cell motility.

Expected outcomes and interpretation

If the level of MscCa expression is directly correlated with cell motility, we expect that overexpressing MscCa in LNCaP cells will increase migration and invasiveness, whereas gene silencing of the MscCa in PC3 tumor cells will decrease migration and invasiveness. It is also possible that overexpression of an MscCa in PC-3 cells would also reduce cell migration by disrupting the optimal levels of stretch induced Ca^{2+} influx required for coordinated disassembly of rear cell adhesions. In this case reduction in external Ca^{2+} may be effective in rescuing cell motility in these cells.

Pitfalls and alternative strategies

The possibility exists that the transient transfection of the hTRPC1 is not efficient enough to result in significant levels of expression in individual cells or in numbers of cells transfected (i.e., as monitored by GFP fluorescence). In this case, PC3 and LNCaP cells will be stably transfected following the procedures used by Shen & Falzon (2003a). Dr. Miriam Falzon, a consultant on this grant, has successfully applied this technique to generate clones of PC3 cells stably transfected with parathyroid-related protein and will assist in these experiments. Another possibility is that heterologous expression of MscCa in the prostate cells may result in an abnormal membrane surface distribution that is disruptive to the coordinated changes in $[Ca^{2+}]_i$ gradients. We will test for such changes in channel surface distribution by multiple patch-clamp recordings (Sect. d, M2). One of our long term goals will be to directly test for polarized MscCa channel surface distribution on individual tumor cells (i.e., by using immunogold labeling EM)

Significance

Over the last few years it has been demonstrated that changes in the expression of specific regulatory molecules critical in cell motility (e.g., integrins) can act as molecular switches to turn-on and turn-off tumor cell migration and invasiveness (Bonaccorsi et al., 2000; Shen & Falzon, 2003b). If we can demonstrate here that regulation of MscCa expression can also act as a molecular switch to prevent tumor cell migration and invasiveness, it will highlight and reinforce the potential of MscCA as a new target for the development of novel therapeutic strategies to prevent the spread of prostate tumor cells.

Methods

M1. Human prostate tumor cell lines: PC3, a highly aggressive, invasive carcinoma cell line, and LNCaP, a noninvasive, poorly tumorigenic cell line, will be used (American type culture collection). Whereas, PC3 cells undergo unstimulated migration, LNCaP cells are only weakly migratory in the absence of stimulatory signals (e.g., serum) (Slack et al., 2001, see Sect. d). Both cells will be grown in the same medium (Ham's F-12 media supplemented with 7% FBS at 37°C in a humidified 95% O_2-5% CO_2 atmosphere. In some experiments cells will be adapted to grow in zero-FBS by subculturing two or three times in reducing concentrations of FBS. Patch-clamp and microscopy studies will be carried out on cells 2-3 days after plating on glass coverslips.

M2. Patch-/pressure-clamp techniques: Standard cell-attached, inside-out, outside-out whole cell patch-clamp recording will be used (Hamill et al., 1981). The pipettes will be made from thin-walled borosilicate glass (World Precision Instruments) with an outside tip diameter of ~2 μm. Immediately prior to patch clamping, a cover slip will be removed from the culture dish and placed in a microscope stage holder. Cells will be visualized with an IM35 Zeiss microscope with fluorescence and Normaski optics. A custom-built pressure-clamp (McBride & Hamill, 1992) will be used to apply pressure and suction protocols to achieve the initial tight seal and stimulate MS channels. To ensure minimal disruption of membrane-CSK interactions and retention of dynamic channel kinetics (adaptation), a gentle-sealing protocol will be followed (Hamill & McBride, 1997); the suction during sealing will be monitored continuously so that it does not exceed 5 mmHg

usually applied for less than 10 s to obtain a gentle seal. The standard pipette solution will contain (in mM): 140 KCl, 10 EGTA (KOH), 10 Hepes (KOH) at pH 7.4. To measure ion selectivity, ion substitutions and ion concentration changes (e.g., Na^+, K^+, Ca^{2+}, Cl^-) on both sides of inside-out patches will be carried out and reversal potentials and current-voltage relations measured according to standard procedures (Hille, 1984). MscCa blockers and activators will be added directly to the pipette solution for cell-attached patch recordings and to the bath for outside-out patch and whole cell recordings. The standard bath solution will contain normal Krebs (in mM) 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (NaOH) at pH 7.4. Patch currents will be filtered at 500 Hz with and 8-pole Bessel filter and digitized at 1 kHz on an IBM clone using pCLAMP (Axon Instruments) acquisition and analysis software packages.

M3. ***Cell migration and invasion assays:*** Cell migration measurements will be carried out using the FluoroBlok system (BD Pharmingen). Cells will be trypsinized, washed once with medium containing 7% FBS, then once in serum-free medium, and resuspended in PBS. After counting, 0.5 x 10^6 cells will be loaded with Calcein-AM (Molecular Probes), and incubated for 2 h in a humidified incubator at 37°C in 95% air/5% CO_2. After centrifugation, the cells will be washed once in FBS-containing medium and once in serum-free medium. After resuspension in serum-free medium, the cells will be plated onto FluoroBlok inserts (BD Pharmingen) that are either uncoated (clear) or coated on their lower surface with laminin or vitronectin (100 ug/ml). Medium containing FBS at varying concentrations (0% -10%) will be added to the lower chamber to test its effects as a chemical attractant. After incubating for 4 or 24 h in a humidified incubator at 37°C in 95% air/5% CO_2, the plates will be scanned using a bottom-reading fluorescence plate reader (excitation wavelength of 485 nm, emission wavelength of 530 nm. Cell migration will be calculated as follows: Cell migration (%) = (total fluorescence intensity in the presence of FluoroBlok insert/total fluorescence intensity in the presence of control insert) x 100. Pilot studies will be carried out to verify a linear and reproducible relationship between cell number and fluorescence intensity. Cell invasion measurements will involve similar procedures except FluoroBlok inserts (BD Pharmingen) will be coated with Matrigel. This reconstituted basement membrane creates a matrix barrier that cells must adhere to and degrade in order to traverse. Matrigel will be thawed out overnight at 4°C, and 100 µl of a 3 mg/ml stock added using a pre-cooled pipette tip onto a pre-cooled FluoroBlok insert mounted onto a pre-cooled 24-well companion plate. The plates will be kept in a humidified incubator at 37°C in 95% air/5% CO_2 for 2 h prior to adding cells. Cells will be prepared and plated as described for the migration assays, and left in contact with the Matrigel for 4h or 24 h. The number of invading cells will be calculated as for the migration experiments. Inhibitors and activators of the MscCa will be added directly to the chambers to study effects on cell migration and invasion. Gd^{3+} will only be studied in the absence of serum.

M4. ***Western Blots:*** Protein samples will be prepared from prostate cell lysates in a buffer containing (mM): 142 KCl, 5 $MgCl_2$, 10 Hepes, 1 EGTA, 1 PMSF, 0.1% protease inhibitors and 1.5% OG at pH 7.2. Membrane protein soubilization will be performed overnight by gentle shaking at 4 °C in the cold room. After centrifugation (13,000rpm for 15 min), supernatants will be stored at -80 °C. The total protein

concentration in the samples will be measured using bicinchonic acid (BCA) assay. For immunobloting studies the protein samples will be resolved in a 8% SDS/PAGE gel, transferred to a PVDF membrane and tested with a commercial anti-TRPC1 antibody (Alomone Labs, Jerusalem, Israel) raised against the peptide QLYDK GYTSK EQKDC corresponding to amino acid residues 557-571 of hTRPC1 (Accession P48995). Incubations with first and secondary antibodies will be performed in 1.5% dry milk T-PBS and blots developed by chemiluminescence. Blocking peptide against the TRPC1 antibody will be used to demonstrate the specificity of the signal. Anti-actin antibody will be used as an internal control for these assays.

M5. TRPC1 Overexpression: The cDNA encoding human TRPC-1, provided by Dr. Craig Montell (Johns Hopkins School of Medicine) (Wes et al., 1995), will be inserted into the dual-function vector, pXOOM, that allows for expression in both mammalian cells and *Xenopus* oocytes (Jespersen et al., 2001). The pXoom vector also includes the neomycin resistance-enhanced GFP fusion protein that makes it possible to visualize transiently transfected mammalian cells by fluorescence microscopy (excitation ~500 nm). To carry out prostate cell transfection, the day before transfection, 2 x 10^6 PC3 or LNCaP cells will be plated in T75 culture flasks. The prostate cells will be transfected with 1 μg plasmid using LIPFECTAMINE PLUS reagent (Invitrogen). Control cells will be exposed to LipofectAMINE alone using the same protocol. Western blotting will be used to confirm the overexpression of TRPC1 in the prostate tumor cells. Patch-clamp recording, cell migration and invasion assays will be performed 48-72 h after transfection, as described above.

M6. Gene silencing by siMRAs: To make full length double-stranded (ds) RNA, two DNA *htrpc1* templates that are identical except with single T7 promotor sites at opposite ends will be constructed. The cDNA templates will be linearized from the vectors and single stranded (ss) RNA made using the MEGAscript kit (AMBION). Equal amounts of the two ssRNA products will be mixed and heated at 60°C and cooled down for 30 min until reaching RT. The transcription template(s) and any ssRNA will be removed by nuclease digestion, and the long dsRNA purified with a solid-phase adsorption system (Transciption Reaction Filter Cartridges). The product will be compared using nondenaturing electrophoresis with both ssRNA products to confirm the presence of dsRNA. Next the long dsRNA will be cleaved with the recombinant *E.coli* RNase III in a 1 h reaction to produce siRNA of 12-30 bp dsRNAs containing a 5 and 3 prime ends and a 2 nucleotide 3' overhand using the Silencer siRNA Cocktail Kit (AMBION). Finally, the siRNA will be purified to remove any undigested or partially digested material. After purification 5 μg of siRNA will be mixed with Lipofectamine plus (Life Technologies) and the mixture placed on 60 mm petri dishes containing confluent PC3 and LNCaP cells. 48 and 72 h later the same cells were transfected again with 5 μg of ssRNA each time. Control cells will be exposed to LipofectAMINE alone using the same protocol. Western blotting will be used to assay the expression of TRPC1 in isolated membrane fractions. Patch-clamp experiments and cell motility assays will be performed 48-72 h after the final transfection.

M7. Cell viability assay: To test whether chemical changes (i.e., channel blockers and serum reduction) and genetic manipulations reduce cell viability, cells will be assayed for increased apoptosis/necrosis, based on phosphatidylserine (PS)

exposure in the external membrane leaflet. Annexin-FITC will be used to label PS externalization and propidium iodide (PI), a nuclear stain that only enters permeabilized cells, will be used to distinguish apoptotic from necrotic cells. PS externalization will be assayed either qualitatively under fluorescence confocal microscopy or quantitatively with FACscan (Becton Dickenson) flow cytometry (FCM) using CellQuest software. Annexin-FITC/PI labeling will be applied to human prostate tumor cells according to the manufacturer's instructions (Boehringer-Mannheim). These techniques have been applied previously in our laboratory to study apoptosis in frog RBCs (Hamill et al., 2000).

Timetable

The experiments in **Specific Aim 1** will be commenced in Year 1 and competed in the first half of Year 2. In Year 1 we will also begin and complete the experiments of **Specific Aim 2.** For **Specific Aim 3,** we have already inserted hTRPC1 in a vector for mammalian cell transfection, so the overexpression experiments will be commenced in Year 1, initially to determine transfection efficiency and effects on cell viability. The patch-clamp characterization of transfected cells will be completed in Year 2. The gene constructs for the siRNAs strategy (Sect d, M6) will be inserted in vectors in Year 1 and the experiment completed in Year 2.

Future Goals

We have several future goals that will extend from the completion of the specific aims of this project. The first will be to identify and clone the endogenous cDNAs for TRPC1 and other TRPCs in prostate tumor cells. Our Western blots indicate that the protein identified as TRPC1 runs with a molecular mass of ~100 kDa, which is larger than both TRPC1α (~92 kDa) and TRPC1β (~80 kDa). Although this difference may reflect posttranslational changes, it may also indicate that prostate tumor cells express specific TRPC1 splice variants, which could open the way for selective targeting of these channels. In addition to the various strategies that have been used to determine TRPC channel subunit stoichiometry (Hofmann et al., 2002), we will also use a novel and potentially more powerful strategy of overexpression, protein purification and reconstitution using patch-clamp assays to examine the effects of specific subunit assemblies on channels properties when reconstituted into a well-defined lipid and protein environment. Our new insight into the structure of the MscCa will also allow us to examine reveal the surface distribution and membrane trafficking of the MscCa in motile cells. In particular, we wish to build on the concept that mechanical forces associated with cell movement regulate the rates of membrane protein insertion and retrieval (trafficking) to maintain a dynamic and polarized distribution of membrane receptors and channels (Sheetz et al., 1999). Our own studies on this subject have already indicated that membrane trafficking (as monitored by ATP release from transport vesicles) is highly MS and integrin-dependent (Maroto & Hamill, 2001).

In summary, the new knowledge of the protein nature of the MscCa provides the first opportunity to use the full arsenal of molecular biological and proteomic techniques to study the mechanisms and role of this channel in regulating cell motility, particularly prostate tumor cell motility. The results of this R21 project will serve as a critical foundation for these future studies.

Example 2

2. Technical Abstract

The stretch-activated Ca^{2+}-permeable channel: a mechanosensory switch for invasiveness of prostate tumor cells

Owen P. Hamill, PhD, PI

Background: Recently, an exciting new concept has emerged that dysregulation of cell motility may be a critical factor in the metastatic cascade leading to invasion by tumor cells. Cell motility involves a coordinated cycle of events including cell extension, adhesion, contraction and release. The cycle is conserved among diverse cell types including keratocytes, fibroblasts and tumor cells. As a consequence, there is strong motivation for identifying regulatory molecules that are rate limiting in cell motility. Recently, the stretch-activated Ca^{2+} permeable channel (SACC) has been proposed to play a critical role in cell motility. This channel transduces membrane stretch into a Ca^{2+} influx, thereby providing feedback between cell extension and Ca^{2+}-dependent release of adhesions. In particular, it has been demonstrated in the rapidly moving fish keratocyte that increased membrane tension in the rear of a forward protruding cell activates the SACC, triggering an influx of Ca^{2+}. The resultant Ca^{2+} transient activates detachment mechanisms that lead to release of the cell rear, allowing the forward movement of the cell. It has also been demonstrated that Gd^{3+}, a commonly used blocker of the SACC, blocks Ca^{2+} transients, inhibits the detachment of the cell, and thereby blocks cell motility. These findings if extended to prostate tumor cells could implicate the SACC as a novel therapeutic target in prevention of prostate tumor metastasis by preventing tumor cell invasion. However, there have been no patch-clamp studies to directly demonstrate SACC expression in prostate tumor cells. Furthermore, interpretation of a report on Gd^{3+} effects on prostate tumor cell motility is problematic because measurements were made in medium containing serum that avidly binds Gd^{3+}. Finally, without identification of the membrane protein(s) that forms the vertebrate SACC(s), it has not been possible to measure SACC protein expression in prostate tumor cells under genetic and environmental conditions that could alter metastatic potential. Our laboratory is well qualified to address all three issues. We have developed patch-clamp/pressure-clamp techniques to characterize the SACC, we are familiar with the use of agents that modulate SACC activity and most recently we have shown, using membrane protein solubilization and functional reconstitution, that in the *Xenopus* oocyte, TRPC1, a member of the Transient Receptor Potential Channel family, is alone sufficient to form the SACC.

Objective/Hypothesis: The objective of this project is to quantify and regulate SACC/TRPC1 expression in prostate tumor cells with markedly different metastatic potential. **The hypothesis to be tested is that the SACC(s) plays a critical role in cell migration and serves as a molecular switch, for prostate cancer invasiveness and metastasis.** To test this hypothesis we will pursue three specific aims:

1. To quantify the functional expression of the SACC(s) in prostate cancer cells with different metastatic potential, using patch-clamp/pressure-clamp techniques.
2. To quantify the expression of membrane protein, TRPC1, a membrane protein shown to form the SACC in other cells.
3. To modify SACC/TRPC1 expression in order to down regulate the invasiveness of prostate tumor cells and thereby their metastatic potential.

Study Design: In **Aim 1**, patch-clamp and pressure-clamp techniques will be used to functionally identify and characterize SACC properties including pharmacology and surface distribution on prostate tumor cell lines with different metastatic potential. In **Aim 2**, immunochemical techniques (Western blots) using specific antibodies against TRPC1 will be used to measure the membrane expression of the putative SACC protein in the different prostate cell lines. In **Aim 3** genetic techniques of heterologous overexpression and gene silencing will be used to alter the TRPC1 expression and study its effects on prostate tumor cell migration.

Relevance of the Proposed Work to the Prostate Cancer Program Goals: A major challenge for treating prostate cancer is to discover molecular markers for and therapeutic targets in metastatic cells. The present study is highly relevant to this goal because it will exploit our recent molecular identification of the SACC to test the hypothesis that the SACC is a mechanosensory switch for prostate cancer invasiveness.

3. PUBLIC ABSTRACT

The stretch-activated Ca^{2+}-permeable channel: a mechanosensory switch for invasiveness of prostate tumor cells

Owen P. Hamill, PhD, PI

Prostate cancer is the most common form of cancer in men. In the early stages, the disease stays in the prostate and is not life threatening, but without treatment it spreads to other parts of the body (metastasizes) and eventually causes death. In the United States, some 40,000 men die every year from prostate cancer that has spread. Current therapies are limited to treatments within the prostate. There is no cure for the disease once it has spread. Therefore, a major challenge is to identify the underlying molecular mechanisms that enable prostate cancer to spread.

Cell migration plays a critical role in the spread of prostate cancer. Tumor cells migrate from the prostate, pass through blood vessels, penetrate the secondary tumor site (typically bone), and migrate through the tissue to establish a secondary metastatic site. Thus, identifying and characterizing the molecules that enhance or suppress cell migration should lead to disease markers and therapeutic targets for prostate cancer.

Cell migration is a multi-step cycle involving extension of a protrusion, formation of stable attachments near the leading edge of the protrusion, translocation of the cell body forward, release of adhesions, and retraction of the cell rear. The force of migration is generated as myosin acts on the actin cytoskeleton (CSK) and pulls against integrin-extracellular matrix (ECM) linkages (focal adhesions). However, for movement to occur, there must be mechanisms that coordinate the assembly and disassembly of focal adhesions at the front and rear of the cell. Several possible candidates have been proposed. An exciting new prospect is the stretch-activated Ca^{2+} permeable channel (SACC) that is gated open as the rear as the cell becomes stretched during extension. The local elevation in internal Ca^{2+} results in disassembly of the focal adhesions and retraction of the cell rear, likely through activation of calpain, a Ca^{2+} sensitive protease. In this case, block or down-regulation of SACC expression may prevent prostate tumor cell invasiveness.

Although the above hypothesis is attractive, a major hindrance to directly testing it at the molecular level has been the unknown identity of the membrane protein(s) forming the vertebrate SACC. Without knowing the membrane protein, regulation of SACC expression, subcellular localization and interactions with other membrane proteins could not be examined in prostate tumor cells. Furthermore, patch-clamp studies are needed in order to directly demonstrate the functional expression of the SACC. The specific aims of this project address these two critical issues. Our first aim is to use patch-clamp and pressure-clamp techniques to determine the functional properties of the SACC in prostate tumor cells with different metastatic potential. Our second aim is to measure the levels of expression of a membrane protein that has recently been implicated in forming the SACC in vertebrate cells. The protein TRPC1, is a member of the Transient Receptor Potential Channel family of Ca^{2+} permeable channels. Our final aim is to directly modulate SACC/TRPC1 expression in order to demonstrate its role in determining tumor cell invasiveness.

PROPOSAL RELEVANCE STATEMENT

The stretch-activated Ca^{2+}-permeable channel: a mechanosensory switch for invasiveness of prostate tumor cells

Owen P. Hamill, PhD, PI

Cell migration is a critical step in prostate tumor metastasis, and the SACC has been implicated in regulating cell migration. Therefore, changes in SACC functional expression may serve as a mechanosensory switch in the transition of prostate tumor cells from the non-motile to the motile and potentially metastatic state. In this case, SACC expression could serve as a marker of and potential therapeutic target against prostate cancer metastasis. However, at this time there have been no patch-clamp studies to directly demonstrate SACC properties and functional expression in prostate tumor cells and the membrane protein(s) that forms the vertebrate the SACC(s) has until recently been unidentified. Our recent studies, based on membrane protein solubilization, purification and reconstititution, have shown in *Xenopus* oocytes that TRP1, a member of the Transient Receptor Potential Channel family, is alone sufficient to form the SACC when reconstituted in liposomes and assayed with patch-clamp recording. The experiments of this project are designed to address the key issue involving the identification and modification of SACC/TRPC1 expression in human prostate tumor cells in order to test their role in prostate tumor cell invasiveness and metastatic potential.

Given that once a prostate tumor metastasizes it invariably proves fatal, it becomes of vital importance to identify the key molecules that may either promote or suppress the transition of the prostate tumor cell from the non-motile to the invasive state. Thus our project, designed to identify the putative mechanosensory switch that regulates this transition, is highly relevant to achieving this goal.

Main body.

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Background

Prostate cancer mortality is a progressive process involving transformation to unlimited cell growth, immortalization to escape the limits of senescence and apoptosis and the ability to grow at distal sites (invasion and metastasis). Prostate tumor cell invasion and metastasis involve (among other things) the transformation of non-motile cells into motile cells capable of migrating to distal sites. Because identifying the most sensitive dysregulated step in this transformation should provide the most promising therapeutic target, there is great interest in understanding the rate-limiting processes in cell motility (Sheetz et al., 1999; Kassis et al., 2001; Wells et al., 2002). In particular, regulatory molecules that are specific to the process of cell motility must be identified. Several events have been implicated in tumor cell transformation involving growth factors, molecules of the extracellular matrix, adhesion receptors, the cytoskeleton, and specific signaling pathways. However, an exciting new prospect is the stretch-activated Ca^{2+} permeable channel (SACC) that has been shown to be critical for several processes including high cell motility ((Guharay & Sachs, 1984; Hamill & McBride, 1992; Lee et al., 1999). In particular, it has been demonstrated in the fast-moving fish keratocyte that SAAC activity mediates the transient elevation in $[Ca^{2+}]_i$ required for the rapid disassembly of cell rear adhesions, thereby allowing directional movement (Lee et al., 1999). If SACC activity is blocked by Gd^{3+}, a commonly used SACC blocker, so is rear detachment and cell movement (Yang & Sachs, 1989; Hamill & McBride, 1996; Lee et al., 1999).

Many studies indicate that cell motility in different cell types shares a highly conserved cycle of events, presumably including SAAC-mediated cell detachment. Thus, an attractive and readily testable hypothesis is that ***SACC activity is rate-limiting in prostate tumor cell motility, and that dysregulation of SACC expression transforms non-motile cells into motile cells, thereby directly contributing to tumor cell invasion and potentially metastasis.*** We have already made good progress into developing the tools to test this hypothesis. However, two major aspects of this model remains to be established. First, although patch-clamp studies indicate SACCs are widely expressed in vertebrate cells, no patch-clamp studies have been done to directly demonstrate the SACC in prostate tumor cells. This is a critical issue, because the generality of the fish keratocyte results has been called into question by a report that Gd^{3+} potentiated, rather than blocked, prostate tumor cell migration (Verrall et al., 1999). However, technical and other concerns make interpretation of this report problematic. First, Gd^{3+} effects on prostate cell migration were measured in medium containing serum, which includes components that avidly bind Gd^{3+} and thereby decrease its blocking efficacy (Hamill & McBride, 1996; Caldwell et al., 1998). Second, in some cells concentration-dependent biphasic (stimulatory and inhibitory) effects of Gd^{3+} on stretch-activated channels have been reported (Hamill & McBride, 1996). Both these concerns indicate the necessity of directly measuring Gd^{3+} effects on both SACC activity and prostate tumor cell migration. Furthermore, because a more potent and specific SACC blocker, GsMtx-4, has recently been purified from the venom of the tarantula *Grammostola spatulata* (Suchyna et al., 2000), it is important that its effects be tested. It is also important to test amiloride analogs that show a characteristic order of potency in blocking SACCs (Lane et al., 1994). Our project will perform these tests on prostate tumor cell motility.

The second major gap in our knowledge, is that the proteins(s) forming the SACCs have not been firmly identified. Only with this knowledge could changes in SACC expression, subcellular localization, and interactions with other membrane proteins be measured under conditions that might lead to acquisition of tumor cell invasiveness. Several putative candidate proteins have been suggested (for review see Hamill & Martinac, 2001). However, our recent studies using membrane protein solubilization and functional reconstitution have shown in the *Xenopus* oocyte that TRPC1, a member of the Transient Receptor Potential Channel family, is alone sufficient to form the SACC when reconstituted in liposomes and assayed with patch-clamp recording. TRPCs are known to form Ca^{2+}-permeable channels that are widely expressed in animal cells and implicated in diverse functions, including receptor- and store-mediated Ca^{2+} influx, volume regulation, pain, and mechanotransduction (Minke & Cook, 2002).

Our group is highly qualified to fill these knowledge gaps. We have contributed directly to the development of patch-clamp/pressure-clamp techniques for studying the SACC (Hamill et al., 1981; McBride & Hamill, 1992; Hamill & McBride, 1995), and have characterized SACC properties in a variety of cell types including the *Xenopus* oocyte and cultured mammalian cells (Hamill et al., 1993; Hamill & McBride, 1996; Hamill & Martinac, 2001). We also have direct experience in identifying the recording conditions that best preserve the stretch sensitivity of the SACC (Hamill & McBride, 1997; Zhang & Hamill, 2000). Finally, based on our recent studies indicating that TRPC1 forms the vertebrate SACC, we have inserted the human form of TRPC1 (Wes et al., 1995) and small interfering RNAs for TRPC1 in a mammalian expression vector to carry out over expression and gene silencing of TRPC1 in prostate tumor cells

Concept and predicted outcome

The concept explored in this project is that the SACC acts as a mechanosensory switch to induce prostate tumor cell motility. The SACC performs this function by its unique characteristics of transducing membrane tension changes into Ca^{2+} influx. This ability allows the SACC to provide the critical link between cell extension/stretch and Ca^{2+}-dependent processes (e.g., calpain activation) that regulates the balance between the disassembly/assembly of cell adhesions required for forward displacement of the cell. For this reason, dysregulation of SACC expression may be a rate-limiting step leading to tumor cell invasiveness.

Our first task is to extend to human prostate tumor cells the pivotal observations of Lee et al (1999) made on fish keratocytes. The predicted outcome is that prostate tumor cells will show SACC expression. We expect this result because patch-clamp studies over the last 20 years indicate that many, if not all cells, express the SACC(s). This includes various mammalian cell lines. The likely reason for this ubiquitous expression is that the SACC has roles in several fundamental mechanosensitive functions including cell motility. A key issue in our study is whether the main difference between motile and non-motile cell arises because of differences in the functional expression and/or surface distribution of the SACC(s) or because of other factors, possibly related to differences in the expression or coupling with downstream Ca^{2+} signaling mechanisms that regulate cell adhesion. Our patch-clamp and immunochemical expression studies of SACC/TRPC1 in the two prostate cell lines with different motilities should make this basic distinction. Similarly, pharmacological and genetic regulation of SACC activity in each cell line, and its effect on cell motility, should directly test whether the SACC represents an effective therapeutic target against transformation of prostate tumor cells into the invasive phenotype.

Objectives: The specific aims of this project are to:

1. To quantify the functional expression of the SACC in prostate cancer cells with different metastatic potential, using patch-clamp and pressure-clamp techniques.
2. To use immunochemical techniques (Western blots) to measure expression of the SACC protein, TRPC, in prostate tumor cells with different metastatic potential.
3. To pharmacologically and genetically silence expression of SACC/TRPC1 in order to arrest the motility and metastatic potential of prostate tumor cells.

Innovation: Our project is innovative because it should identify and characterize a critical molecule in prostate tumor cells that has been shown to rate limiting in cell motility. Our study will represent the first patch-clamp characterization of the SACC in prostate tumor cells and the first study identifying and modifying the expression of the SACC/TRPC1 protein in order to test its role in determining prostate tumor cell invasiveness.

Relevance: Current prostate cancer treatments focus on blocking cell proliferation and/or cancer cell immortalization. However, given that once a prostate tumor metastasizes it invariably proves fatal, it is vitally important to identify the key molecules that may either promote or suppress the transformation of the prostate tumor cell from the non-motile to the invasive state. Thus our project, designed to identify a novel mechanosensory switch that could regulate this crucial transformation, is an important step to achieving this goal.

Abbreviations

Stretch-activated Ca^{2+} permeable channel:	SACC
Transient receptor potential channel:	TRPC
Cytoskeleton:	CSK
Extracellular matrix:	ECM
Intracellular Ca^{2+} concentration:	$[Ca^{2+}]_i$

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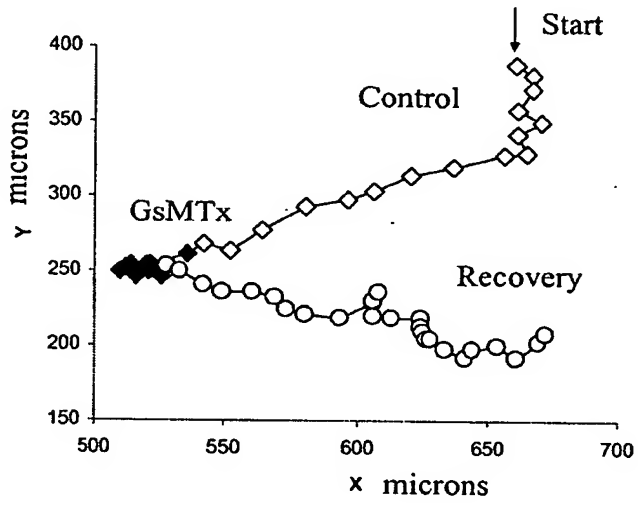
Example 3

We have tested the Tarantula venom peptide on migration of human prostate tumor cells. The following Figure shows the migratory path of a single human prostate PC3 tumor cell starting at the arrow in the presence of control saline (◇), after addition of 100 nM GsMtx (◆) and following washout of the GsMTx (O)



The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A method for treating metastatic cancer comprising:
administering to a subject having prostate cancer an effective amount of a composition comprising an agent that binds to a stretch-activated Ca^{2+} permeable channel (SACC) present on a prostate cancer cell surface.

2. The method of claim 1 wherein the agent is a polypeptide comprising an amino acid sequence as depicted at Genbank accession number A59371, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number A59371.

3. The method of claim 1 wherein the agent is an antibody.

4. The method of claim 1 wherein the SACC comprises an amino acid sequence as depicted at Genbank accession number CAA61447, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number CAA61447.

5. A method for decreasing invasion by a cancer cell comprising:
administering to a subject having cancer an effective amount of a composition comprising an agent that binds to an SACC present on the cancer cell surface.

6. The method of claim 5 wherein the agent is a polypeptide comprising an amino acid sequence as depicted at Genbank accession number A59371, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number A59371.

7. The method of claim 5 wherein the agent is an antibody.

8. The method of claim 5 wherein the SACC comprises an amino acid sequence as depicted at Genbank accession number CAA61447, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number CAA61447.

9. A method for identifying an agent that decreases migration of a cancer cell comprising:
contacting a motile cancer cell with an agent that binds to an SACC present on the cancer cell surface; and
observing motility of the cancer cell, wherein a decrease in motility of the cancer cell compared to a cancer cell not contacted with the agent indicates the agent decreases migration of the cancer cell.

10. The method of claim 9 wherein the SACC comprises an amino acid sequence as depicted at Genbank accession number CAA61447, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number CAA61447.

11. The method of claim 9 wherein the motile cancer cell is a human prostate tumor cell line.

12. The method of claim 11 wherein the human prostate tumor cell line is ATCC CRL-1435 (PC-3).

13. The method of claim 9 wherein the motile cancer cell is present in a subject.

14. An agent identified by the method of claim 19.

15. A method for decreasing motility of a cancer cell comprising:

contacting a motile cancer cell with an agent that binds to an SACC present on the cancer cell surface; and

observing motility of the cancer cell, wherein a decrease in motility of the cancer cell compared to a cancer cell not contacted with the agent indicates the agent decreases migration of the cancer cell.

16. The method of claim 16 wherein the SACC comprises an amino acid sequence as depicted at Genbank accession number CAA61447, or an amino acid sequence having at least about 90% identity with the amino acid sequence depicted at Genbank accession number CAA61447.

17. The method of claim 16 wherein the motile cancer cell is present in a subject.

18. A method for treating metastatic cancer comprising:

administering to a subject having prostate cancer an effective amount of a composition comprising a polynucleotide comprising at least about 15 consecutive nucleotides from the nucleotide sequence depicted at Genbank accession number X89066, or the complement thereof.

19. The method of claim 18 wherein the polynucleotide is present in an expression vector.

20. A method for decreasing invasion by a cancer cell comprising:

administering to a subject having cancer an effective amount of a composition comprising a polynucleotide comprising at least about 15 consecutive nucleotides from the nucleotide sequence depicted at Genbank accession number X89066, or the complement thereof.

21. The method of claim 20 wherein the polynucleotide is present in an expression vector.

22. A method for decreasing motility of a cancer cell comprising:

introducing to a motile cancer cell a polynucleotide comprising at least about 15 consecutive nucleotides from the nucleotide sequence depicted at Genbank accession number X89066, or the complement thereof; and

observing motility of the cancer cell, wherein a decrease in motility of the cancer cell compared to a cancer cell not contacted with the agent indicates the agent decreases migration of the cancer cell.

23. The method of claim 22 wherein the polynucleotide is present in an expression vector.